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Title: Circulating metabolites and lipids are associated to diabetic retinopathy in individuals with type 1 diabetes

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Abstract

Omics based methods may provide new markers associated to diabetic retinopathy (DR). We investigated a wide omics panel of metabolites and lipids related to DR in type 1 diabetes. Metabolomic analyses were performed using two-dimensional gas chromatography with time-of-flight mass spectrometry and lipidomic analyses utilizing an ultra-high-performance liquid chromatography quadruple time-of-flight mass spectrometry method in 648 individuals with type 1 diabetes. Subjects were sub-divided into: no DR, mild non-proliferative DR (NPDR), moderate NPDR, severe NPDR and proliferative DR. Endpoints were any progression of DR, onset of DR and progression from mild to severe DR tracked from standard ambulatory care and investigated using Cox models. The cohort consisted of 648 participants aged mean 54.4±12.8 years, 55.5% were male, and follow-up was 5.1-5.5 years. Cross-sectionally, 2,4-dihydroxybutyric acid (DHBA), 3,4-DHBA, ribonic acid, ribitol, and the triglycerides 50:1 and 50:2 significantly correlated ($p<0.042$) to DR stage. Longitudinally, higher 3,4-DHBA was a risk marker for progression of DR (n=133) after adjustment ($p=0.033$). We demonstrated multiple metabolites being positively correlated to higher grade of DR in type 1 diabetes, and several triglycerides being negatively correlated. Furthermore, higher 3,4-DHBA was an independent risk marker for progression of DR, however, confirmation is required.

Introduction

One of the most frequent and debilitating complications of diabetes is diabetic retinopathy (DR). Despite radical improvements in diagnosis and treatment throughout the last decades (1), DR is still the primary cause of blindness in individuals with diabetes aged 20-74 years, and the prevalence was 34.6% in a large meta-analysis including 22,896 individuals with diabetes (2). The advances in diagnosis and treatment have particularly been made for later stages of the disease and robust and specific risk markers for onset and early progression of DR is still lacking.

Novel omics methods have been developed, allowing for simultaneous evaluation of large panels of metabolites using mass-spectrometry-based approaches that allows for comprehensive study of metabolic pathways compared to more traditional single-biomarker approaches. Omics facilitates advanced and detailed analysis faster than standard methods. More specifically, metabolomics and lipidomics is the analysis and categorization of circulating metabolites and lipids using this method; and can provide a more comprehensive view of various metabolites' biological effects (3, 4).

Applying this method to DR is an intriguing concept. Omics allows for a unique ability to understand the biological pathways for DR, as well as facilitate the discovery of novel biomarkers associated to development and progression. Few studies have evaluated the association between circulating metabolites and presence of DR. In one cross-sectional study, several metabolites were associated with DR in type 2 diabetes, amongst them, hydroxyl fatty acids, such as 3,4 dihydroxybutyric acid and sugar derivatives such as lactose, maltose and ribose (5). Likewise, other studies have identified plasma metabolites associated to arginine-, pyrimidine- and fatty acid-related pathways, as well as several amino acids highly related to insulin resistance, in relation to DR (6-8).

In the present study we investigated the predictive qualities of a wide panel of metabolites and lipids in plasma in relation to the presence, onset and progression of DR in individuals with type 1 diabetes.

Methods

Study population

Between 2009 and 2011, 648 individuals with T1D and a large range of albuminuria were recruited from the outpatient clinic at Steno Diabetes Center Copenhagen. The details of the cohort have previously been described (9). Participants were subdivided by stages of albuminuria (normo-, micro- and macroalbuminuria). End stage kidney disease, defined as receiving dialysis, renal transplantation or glomerular filtration rate (GFR) <15 ml/min/1.73m^2 at baseline was an exclusion criterium. In the present study, metabomics and lipidomics data along with information on retinopathy status was available for 601 (serum metabolomics) and 648 (plasma lipidomics) participants, respectively.

The study was conducted in compliance with the Declaration of Helsinki and was approved by the local ethics committee. All participants have given informed written consent.

Baseline clinical analyses

Serum creatinine, plasma LDL-cholesterol, triglycerides and HbA_{1c} were measured using standardized methods from venous samples. Urinary albumin excretion rate (UAER) was analyzed by enzyme immunoassay based on three consecutive 24h urine collections. eGFR was calculated

based on serum creatinine using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Sitting brachial blood pressure was measured after 10 minutes rest using an automated validated device.

Baseline DR stage was classified using in-house algorithms on a 0-4 scale based on regular retinopathy screenings at Steno Diabetes Center Copenhagen performed by specifically trained and certified nursing staff under supervision of ophthalmologists. Five mydriatic non-stereoscopic fundus photos are taken; one macula-centered and four peripheral 45° fundus images. These are combined into a mosaic, grading the macula and periphery separately, according to a modified version of the International Classification of diabetic retinopathy disease severity scale (10). The presence of microaneurysms, intraretinal hemorrhages, hard- or soft exudates and proliferations are recorded and quantified. Likewise, intraretinal microvascular abnormalities and venous beading are recorded. Based on a weighted quantitation of the distinct retinal pathologies, staging was performed. Overall stage was defined as the highest stage diagnosed in either eye. Stage 0 is defined as no DR in any eye, stage 1 as mild non-proliferative retinopathy (NPDR), stage 2 as moderate NPDR, stage 3 as severe NPDR and stage 4 as proliferative retinopathy (PDR). Blind subjects are not screened for retinopathy at Steno Diabetes Center Copenhagen and were therefore excluded. Blindness was defined as visual acuity of less than 1/60, lack of ability to count fingers in front of a white screen at 1 m distance, and lack of ability to see hand motion in front of a white screen at 1 m distance.

Sample quantification and identification

The metabolomics and lipidomics analysis are detailed in Tofte et al. (11) and (12), respectively. For completeness, the analyses are outlined here as follows: Serum samples, stored at -80 °C, were

analyzed by two different analytical methods. Metabolomics samples were analyzed using a two-dimensional gas chromatography with time-of-flight mass spectrometry. Peak-picking from the raw data was performed with ChromaTOF, and the resulting features were aligned with Guineu (13).

Lipidomics samples were prepared using a modified Folch extraction procedure (14), and analyzed by a previously presented ultra high-performance liquid chromatography quadrupole time-of-flight mass spectrometry method (UHPLC-Q-TOF-MS) (15). The raw data were pre-processed with MZmine 2 (16). A complete list of identified metabolites is available in Tofte et al. (11). Finally, the metabolomics and lipidomics data were post-processed in R, as described previously (11, 12). Lipid species are defined as number of carbon atoms (indicating total fatty acid chain length) and number double-bonds for the specific species. They are presented as "Species(number of carbon atoms:number of double-bonds)".

Within the coverage of the two mass spectrometry platforms, the inclusion of metabolites and lipids in subsequent data analysis was solely based on the certainty of identification and the level of technical precision, thereby not restricting to any particular pathway or prior hypothesis.

Follow-up

Data regarding retinopathy were obtained using local electronic records from Steno Diabetes Center Copenhagen up to December 31st, 2016 and was available for 563 subjects.

The endpoints were defined as 1) progression from any stage to any other stage of DR (any progression); 2) onset of DR; and 3) progression from stage 1-2 to stage 3-4 (progression from mild to severe DR).

In the case of participants experiencing multiple endpoints, only the first occurrence was included.

Statistical analysis

Continuous variables are presented as mean ± standard deviation, if normally distributed and as median (interquartile range (IQR)) if skewed. Prior to all analyses, skewed variables were log2 transformed, including all metabolites, to achieve normal distribution. Categorical variables are presented as total number (%). Baseline clinical characteristics were compared across baseline DR status using analysis of variance and χ^2-test for continuous and categorical variables, respectively.

Metabolites and lipid species were analysed using a narrowing-down approach in relation to DR stages and outcomes as follows: Cross-sectional relationship between single metabolites or lipid species and baseline DR stages were assessed using multivariate linear regression models adjusted for relevant clinical variables. Thereafter, the single measures were cross-sectionally associated to categories of DR stage and tested using ANCOVA. The Benjamini-Hochberg method (p_{BH}) (17, 18) was used to correct for multiple testing for presented P-values throughout the analysis. Metabolites with $p_{BH}<0.05$ and lipids with $p_{BH}<0.1$ in the adjusted cross-sectional model were included in survival analysis with the Cox proportional hazards model for onset or progression from mild to severe DR of DR. All HRs reported per doubling of metabolite or lipid.

Clinical variables in the adjusted models were age, sex, HbA_{1c}, systolic blood pressure, smoking, body mass index, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication.

Partial correlation network analysis was done with the R-package “huge” (19) using the graphical LASSO algorithm (20) coupled with the extended Bayesian information criterion (21). All metabolites were included in inferring the network. Subsequently, the subnetwork of metabolites, which were immediately connected to the four retinopathy-associated metabolites, were visualized

with the R-package qgraph (22). Edges or the network were colored by the respective partial correlation in the graphical LASSO model, and nodes were colored with respective Spearman correlation to the top candidate biomarkers. In both color annotations, red and blue refer to positive and negative correlation, respectively. Size of nodes refers to the respective degree (i.e., the number of associations to other nodes). Statistical analysis and data visualization were performed using R (version 3.4.2)

Data and resource availability

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Results

Baseline characteristics

Baseline characteristics for the participants divided according to baseline DR stages is shown in Table 1. The majority had no DR (n=141, 22%) or moderate NPDR (n=186, 29%). When comparing to no DR, participants with PDR had higher mean (SD) BMI (24.7 (3.5) versus 26.8 (11.2) kg/m^2), UAER (11.5 (IQR: 7.6-22.4) versus 49.0 (IQR: 14.9-231.8) mg/24h) and systolic blood pressure (127 (46.1) versus 135 (20.3) mmHg), and lower eGFR (94.8 (24.7) versus 64.5 (30.6) ml/min/1.73 m^2). Across groups age, systolic blood pressure and UAER was higher with higher DR stage, and the eGFR was lower. Also, the frequency of treatment with antihypertensive drugs and statins was higher with higher DR stage.

Metabolomic cross-sectional analyses

A total of 75 metabolite species were identified and passed quality control (Supplementary Table 1). These metabolites were included in the multivariate linear regression models. Four metabolites were positively correlated to baseline DR stage after adjustment for clinical variables and correction for multiple testing: 2,4-dihydroxybutyric acid (2,4-DHBA; $p_{BH}<0.001$), ribonic acid ($p_{BH}=0.017$), ribitol ($p_{BH}=0.032$) and 3,4-DHBA ($p_{BH}=0.036$). Thereafter, all 75 metabolites were included in ANCOVA for baseline DR stages: the same four metabolite levels were significantly increased by higher DR stage after adjustment (ribonic acid, $p_{BH}<0.001$; 2,4-DHBA, $p_{BH}<0.001$; ribitol, $p_{BH}=0.013$ and 3,4-DHBA $p_{BH}=0.041$ - Table 2). Figure 1 illustrates the distribution and relative levels of these four metabolites. It is apparent that the levels of all four metabolites increase with higher DR stage. Both, 2,4-DHBA and 3,4-DHBA (Figure 1 B and D), follow the same pattern of lower levels of the metabolite for no DR and NPDR, and a non-linear increase towards higher DR stages and PDR, in particular. In contrast, the levels of ribonic acid and ribitol (Figure1 A and C) increase linearly with increasing DR stage.

The partial correlation network of the metabolome connected to the four metabolites is shown in Figure 2. This network included kidney function-related metabolites (such as creatinine and myo-inositol), glucose metabolism-related compounds (such as citric acid and glycine), fatty acids (such as fumaric acid and malic acid) and amino acids (such as alanine and serine). Particularly creatinine and myo-inositol, but also glyceryl-glycoside, 4-hydroxybenzeneacetic acid and fumaric acid, were highly associated with the hub of the four retinopathy-associated metabolites. Except for the amino

acids, most of the compounds in the network were positively correlated with the four highlighted metabolites, as indicated by red color in Figure 2.

Lipidomic cross-sectional analyses

After identification and quality control, a total of 104 lipid species from the following five major lipid classes were included in the analyses: diacyl-phosphatidylcholines (PCs), alkyl-acyl-phosphatidylcholines (PC-Os), lyso-phosphatidylcholines (LPCs), triacylglycerols (TGs) and sphingomyelins (SMs). Lipid species are defined as number of carbon atoms (indicating total fatty acid chain length) and number double-bonds for the specific species. They are presented as "Species(number of carbon atoms:number of double-bonds)". The investigated lipids are listed in Supplementary Table 2.

Two triacylglycerols TG(50:1) and TG(50:2) were inversely associated with DR grade at baseline after adjustment for clinical covariates, when testing with linear regression analysis ($p_{BH}<0.05$). Furthermore, LPC(16:1), PC(32:1), TG(14:0/16:0/18:1), TG(50:3) and PC(32:2) were inversely associated with baseline DR grade at a higher false discovery rate of 10 %.

When investigating with ANOVA, a difference in the lipid level between the DR grades was detected in lyso-phosphatidylcholine LPC(16:0) ($p_{BH}<0.1$). This association was lost after adjustment for the clinical covariates (ANCOVA). In addition to LPC(16:0), medium-sized unsaturated TGs and small LPCs had stronger indicative associations with DR grade than other lipids, as shown in the lipidome-wide heatmap of the ANCOVA F-statistics (Figure 3). In particular, LPCs(16:1) as well as TGs 49:3, 50:1 and 50:2 emerged with an indicative association with the DR grade.

Longitudinal analyses

Metabolites and lipids identified in cross-sectional analyses were thereafter analyzed with compound-specific Cox proportional hazards models for association to any progression, onset of DR, and progression from mild to severe DR. Median follow-up ranged between 5.1-5.5 years depending on endpoint. The number of events were 133, 47, and 29 for any progression, onset of DR and progression from mild to severe DR respectively. For the any progression endpoint, higher 3,4-DHBA exhibited significance after adjustment for clinical covariates and multiple testing (HR (CI 95%):1.55 (1.12-2.15), p=0.033). The other metabolites were not associated with any of the endpoints neither before nor after adjustment. Although not statistically significant, 2,4-DHBA showed a high HR for progression from mild to severe DR (HR (CI 95%): 1.92 (0.94-3.93), p=0.290). Unadjusted and adjusted HRs for the metabolites are presented as a forest plot in Figure 4.

Unlike the metabolites, none of the lipids were independent risk factors for any of the endpoints ($p>0.05$).

Discussion

The present study illustrates an exciting new avenue in characterizing T1D individuals with DR. In the present cohort we have investigated individuals with long diabetes duration and a broad range of albuminuria, leading to a high proportion of subjects with more severe DR than would be expected in a general clinical population with T1D. We identified four metabolites associated with presence of DR, as well as higher 3,4-DHBA as an independent risk marker for progression of DR. Our

results were independent of a panel of metabolic risk factors traditionally used for risk stratification of DR in T1D. Therefore, we now argue for the need for further investigation of omics-based risk stratification of DR. Using omics in relation to DR is a relatively new venture and clinical studies assessing its viability are sparse, especially in subpopulations such as individuals with T1D, and using longitudinal data.

The metabolites identified in this study mainly stem from two etiopathogenic factors, namely hyperglycemia (ribitol and ribonic acid) and dyslipidemia (2,4- and 3,4-DHBA) (23). Ribitol and ribonic acid are derivatives from ribose which is highly active in the pentose phosphate pathway, in the production of nucleotides and nucleic acids. Furthermore, sugar alcohols such as sorbitol, are active in the polyol pathway which has been identified as a crucial insulin-independent pathway relevant in the onset of DR, and have been suggested as a possible therapeutic target in the treatment of DR (24). The fructose created in this pathway becomes further phosphorylated resulting in the formation of advanced glycation end products (AGEs) which in turn bind to receptors for AGEs (RAGE) - a known facilitator of DR (25).

DHBAs on the other hand, which are closely related to the ketone body hydroxybutyric acid, have not been directly associated with any major pathways implicated in the onset of DR or T1D. Other diseases, such as succinic semialdehyde dehydrogenase deficiency, an autosomal recessive genetic disease, leads to 4-DHBA aggregation, and, in turn, is associated to severe neurological complications and symptoms (26). In general, ketone bodies are associated with dyslipidemia and high fat diets (27), which are highly relevant risk factors in the development of diabetic complications. A theory that has drawn recent attention is that DHBAs could be implicated in the butyrate metabolism by the gut microbiota (28), although at present this still calls for more investigation. Similarly, Sumarriva et al. (6) demonstrated that higher plasma carnitine, a metabolite highly present in meat-containing food, was associated to the presence of PDR compared

to NPDR. Carnitine is further metabolized by the gut microbiota, into trimethylamine-N-oxide that has been associated to cardiovascular and metabolic diseases (29).

Interestingly, visualized in Figure 2, the sub-network of metabolites associated with DR, show that all metabolites which correlated significantly with DR in this study, all seem strongly associated to myo-inositol. Despite myo-inositol itself not being associated to DR, in this study, the association to the other metabolites could propose another pathway of DR etiology. Myo-inositol is a sugar alcohol which gastrointestinal absorption and intracellular transport has been shown to be impaired in individuals with diabetes (during hyperglycemia) (33). Furthermore, while previously thought mainly to be expressed in renal tissue, recent studies show evidence of myo-inositol activity in extra-renal tissue such as retinal and lens epithelium as well (34, 35), and has additionally been associated to existing DR in individuals with type 2 diabetes (36).

Our metabolomic results stand well in relation to a study by Chen et al., describing similar metabolites associated to DR in a cross-sectional study including type 2 diabetes individuals with DR (n=40) or without DR (n=40) in the Singaporean Indian Eye Study. They demonstrated that 3,4-DHBA as well as ribose were significantly higher in the DR group compared to the one without. Furthermore, similar results were found for 2-deoxyribonic acid as well as for several other sugars and sugar derivates (5). As such, we can partly validate these results in our larger population of T1D individuals, and additionally, we have shown that 3,4 DHBA was a risk marker for progression of DR during follow up. The inherent issue with omics discovery studies being explorative, is that replication of results is necessary across populations and cohorts, but often difficult, especially due to platform heterogeneity. The study by Chen et al. strengthens the findings in our study suggesting that 3,4 DHBA and ribose derivatives are valid risk markers of DR. However, Lin et al. showed that branched-chain, aromatic and glucogenic amino acids such as leucine, valine, tyrosine and alanine was positively associated with diabetic microangiopathy in

type 2 diabetes. These amino acids were also investigated in our study; however, we could not confirm the results, possibly due to the heterogeneity between type 1 and type 2 diabetes.

In the case of lipids, some, such as LDL cholesterol and TGs, have throughout many years been comprehensively studied and have proved to be robust risk markers for vascular disease in T1D. In addition, studies targeting dyslipidemia with fibrates have found beneficial effects on retinopathy (37, 38). Therefore, the concept of applying lipidomic strategies into finding novel markers to strengthen the identification and prediction of vascular risk, are not implausible. In our panel of lipids, we were only able to identify three TGs negatively correlated with DR stage in our linear models using a 5% α-level, adjusted for i.a. baseline LDL-cholesterol, triglycerides and body mass index. However, these associations could not be replicated in ANCOVA models, arguably in part due to non-linear trends for different lipids across DR stages.

It is difficult to compare the present results in relation to other studies as very few have investigated DR. One study found stearic acid, trans-oleic acid, linoleic acid, arachidonic acid and free cholesterol in circulation to be significant differentiators between preclinical DR, NPDR and PDR (39). Likewise, Schwartzman et al. identified several free-fatty acid autacoids in vitreous humor as markers of PDR in T1D (40).

Proliferative DR is demonstrated to be associated to an impaired blood-brain barrier (BBB), and early damage to the BBB is hypothesized to be a predictor of progression to more advanced stages of DR (41). However, the mechanisms for the association surrounding BBB impairment and DR are not understood, and the association to circulating biomarkers has not been described. Hogan et al. demonstrated that various polyunsaturated fatty acids and sphingolipids was associated with impaired BBB in traumatic brain injury in rats (42), but our results do not support the association between these and DR.

Moving outside of purely metabolomic and lipidomic studies, a substantial amount of research has been performed on the proteome and its effect on DR risk and risk progression, however primarily in small samples with largely non-replicated results (43).

This study is not without limitations. The DR staging during follow up did not take laser surgery or VEGF injections into account, as it was based only on changes in DR stage from baseline. Furthermore, no data on concomitant medication throughout the follow-up was available, and as such there is no data on how statins or antihypertensive medication, or insulin treatment have changed during follow up. In addition, no information on lifestyle parameters, which could have influence on lipid composition, were available at baseline. Finally, the lack of a validation population is another limiting factor. Nonetheless, the sizable strengths of this study are firstly the large, well-defined cohort of individuals with T1D, including 7 years of longitudinal data, and secondly a comprehensive metabolomic and lipidomic analysis regarding presence of and changes in DR in T1D.

In summary, we identified four metabolites and three lipids with an association to the DR stage: ribonic acid, ribitol, two dihydroxybutyric acids were associated with DR stage and three triglycerides were negatively correlated with the DR stage. Furthermore, we have identified the 3,4-dihydroxybutyric acid as an independent risk marker for progression in DR stage. Our results may serve as a basis for further studies regarding sugar metabolism, hydroxy acids and lipids in relation to diabetic complications such as retinopathy, as more investigative studies are needed before these markers can be clinically applied.

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Tables

Table 1: Baseline characteristics across diabetic retinopathy stage.

	No DR	Mild NPDR	Moderate NPDR	Severe NPDR	PDR	p
n (%)	141 (21.8)	90 (14.0)	186 (28.8)	121 (18.8)	107 (16.6)	-
Men, n (%)	69 (49.3)	47 (52.2)	120 (64.5)	62 (51.7)	60 (56.1)	0.051
Age, years	46.5 (15.1)	54.9 (11.8)	56.4 (10.7)	59.1 (10.8)	55.8 (10.9)	<0.001
Diabetes duration, years	16.8 (15.5)	32.1 (16.1)	33.1 (12.4)	41.4 (10.8)	41.3 (9.0)	<0.001
Smokers, n (%)	29 (20.6)	17 (18.9)	46 (24.7)	20 (16.5)	21 (19.6)	0.495
BMI, kg/m^2	24.7 (3.5)	24.3 (3.7)	25.8 (4.3)	25.8 (3.8)	26.8 (11.2)	0.014
HbA_{1c}, mmol/mol	64.3 (14.7)	61.7 (11.2)	63.9 (11.3)	66.0 (13.8)	65.5 (11.4)	0.130
HbA_{1c}, %	8.0 (1.3)	7.8 (1.0)	8.0 (1.0)	8.2 (1.3)	8.1 (1.1)	0.130
eGFR, ml/min/1.73m^2	95 (25)	91 (23)	89 (24)	75 (27)	64 (31)	<0.001
UAER, mg/24h	12 (8-22)	9 (6-24)	15 (8-53)	29 (9-122)	49 (15-232)	<0.001
SBP, mmHg	127 (16)	129 (15)	132 (16)	134 (18)	135 (20)	0.001
Antihypertensive treatment, n (%)	58 (41.4)	49 (54.4)	138 (74.2)	109 (90.1)	103 (96.3)	<0.001
Total cholesterol, mmol/l	4.7 (0.8)	4.7 (0.8)	4.7 (0.9)	4.7 (0.9)	4.6 (0.9)	0.673
Triglycerides, mmol/l	0.9 (0.7-1.3)	0.9 (0.7-1.1)	1.0 (0.8-1.4)	0.9 (0.7-1.3)	1.0 (0.7-1.5)	0.055
Statin treatment, n (%)	54 (38.6)	44 (48.9)	113 (60.8)	92 (76.0)	81 (75.7)	<0.001

Data are mean (standard deviation) or median (inter-quartile range), HbA_{1c}: Glycated hemoglobin, eGFR: Estimated glomerular filtration rate, UAER: Urinary albumin excretion rate, SBP: Systolic blood pressure, DR: Diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy. P-values was calculated using analysis of variance and χ^2-test for continuous and categorical variables, respectively.

Table 2: Cross-sectional association between metabolites and lipids and baseline diabetic retinopathy (DR) grade.

Association to DR grade (MLR)	**Effect**	**CI (95%)**	**p**	**p_{BH}**
Metabolites				
2,4-DHBA	0.097	0.058;0.135	<0.001	<0.001
Ribonic acid	0.109	0.049;0.170	<0.001	0.017
Ribitol	0.072	0.028;0.116	0.001	0.032
3,4-DHBA	0.059	0.022;0.097	0.002	0.036
Lipids				
TG(50:2)	-0.066	-0.104;-0.028	<0.001	0.042
TG(50:1)	-0.074	-0.118;-0.030	0.001	0.042
PC(32:2)	-0.085	-0.140;-0.029	0.003	0.083
LPC(16:1)	-0.087	-0.146;-0.028	0.004	0.084
TG(14:0/16:0/18:1)	-0.065	-0.109;-0.021	0.004	0.084
TG(50:3)	-0.053	-0.091;-0.016	0.006	0.092
PC(32:1)	-0.080	-0.137;-0.023	0.006	0.092

Association to DR grade (ANCOVA)	**F**	**p**	**p_{BH}**
Metabolites			
Ribonic acid	7.44	<0.001	<0.001
2,4-DHBA	6.58	<0.001	0.001
Ribitol	4.98	<0.001	0.013
3,4-DHBA	4.18	<0.001	0.041
Lipids			
TG(50:1)	3.97	0.003	0.223
TG(50:2)	3.55	0.007	0.257
TG(49:3)	3.44	0.009	0.257
LPC(16:1)	3.12	0.015	0.354
LPC(16:0)	2.71	0.030	0.544
TG(52:2)	2.62	0.034	0.544
TG(14:0/16:0/18:1)	2.58	0.036	0.544
PC(32:1)	2.44	0.046	0.547
TG(16:0/18:0/18:1)	2.37	0.052	0.547
PC(32:2)	2.29	0.059	0.547

Table 2: Presented in the top half are multivariate linear regression model effect sizes per increase in DR grade, for each metabolite or lipid to baseline DR grade with 95% CI and crude and Benjamini-Hochberg adjusted p-values. In the bottom half of the table are ANCOVA F-values presented for each metabolite and lipid to baseline DR grade with crude and adjusted p-values. All presented models include the following baseline covariates: age, sex, glycated hemoglobin, systolic blood pressure, smoking, body mass index, statin treatment, triglycerides, low-density lipoprotein cholesterol, and prescribed antihypertensive medication. MLR: Multivariable linear regression, p_{BH}: Benjamini-Hochberg adjusted p-value, DHBA: Dihydroxybutyric acid, TG: Triacylglycerol, LPC: Lyso-phosphatidylcholine, PC: Diacyl-phosphatidylcholine

Figure Legends

Figure 1:
Violin plots showing metabolite levels across baseline diabetic retinopathy stages. Shown are four metabolites, where a difference between the stages was detected using ANCOVA. Observations are shown as dots and their distribution in each of the stages of retinopathy as a violin geom. Pairwise differences between the stages are indicated with p-values at the top-part of the figure. The results are from models with adjustment for age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. A: ribonic acid, B: 2,4-dihydroxybutyric acid, C: ribitol, and D: 3,4-dihydroxybutyric acid, HbA_{1c}: glycated hemoglobin, DR: Diabetic retinopathy, NPDR: Non-proliferative DR, PDR: Proliferative DR

Figure 2:

Partial correlation network showing associations in the measured metabolome. Metabolites with cross-sectional association to retinopathy (ribitol, ribonic acid, 2,4-dihydroxybutyric acid and 3,4-dihydroxybutyric acid) are indicated as diamonds-shaped nodes. The node color of the other metabolites (circlular nodes) indicates the Spearman correlation to ribonic acid. Partial correlations (i.e., independent associations) are indicated by lines between the metabolites, where thickness and color of the line, respectively, indicate the strength and the sign of the association (red: positive, blue: inverse). Additionally, size of the node indicates the degree of the node (i.e., the number of associations with the metabolite).

Figure 3:

Heatmaps of the ANCOVA model F-statistics across the entire lipidomic panel in relation to diabetic retinopathy stage. The results are from models with adjustment for age, sex, HbA_{1c}, systolic blood pressure, smoking, body mass index, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. Lipid species are grouped according to the lipid classes in each panel. Each cell represents one lipid species. On the y-axis is number of double-bonds for the specific species (indicating level of saturation) and on the x-axis is the number of carbon atoms (indicating total fatty acid chain length).

Figure 4:

Forest plot of multiple-testing-corrected hazard ratios of the selected metabolites for three retinopathy events: any retinopathy, onset of retinopathy, and progression from mild to severe retinopathy(top, middle and bottom panels, respectively). Adjusted model (right) is adjusted for age,

sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. Crude model (left) is without these adjustments.

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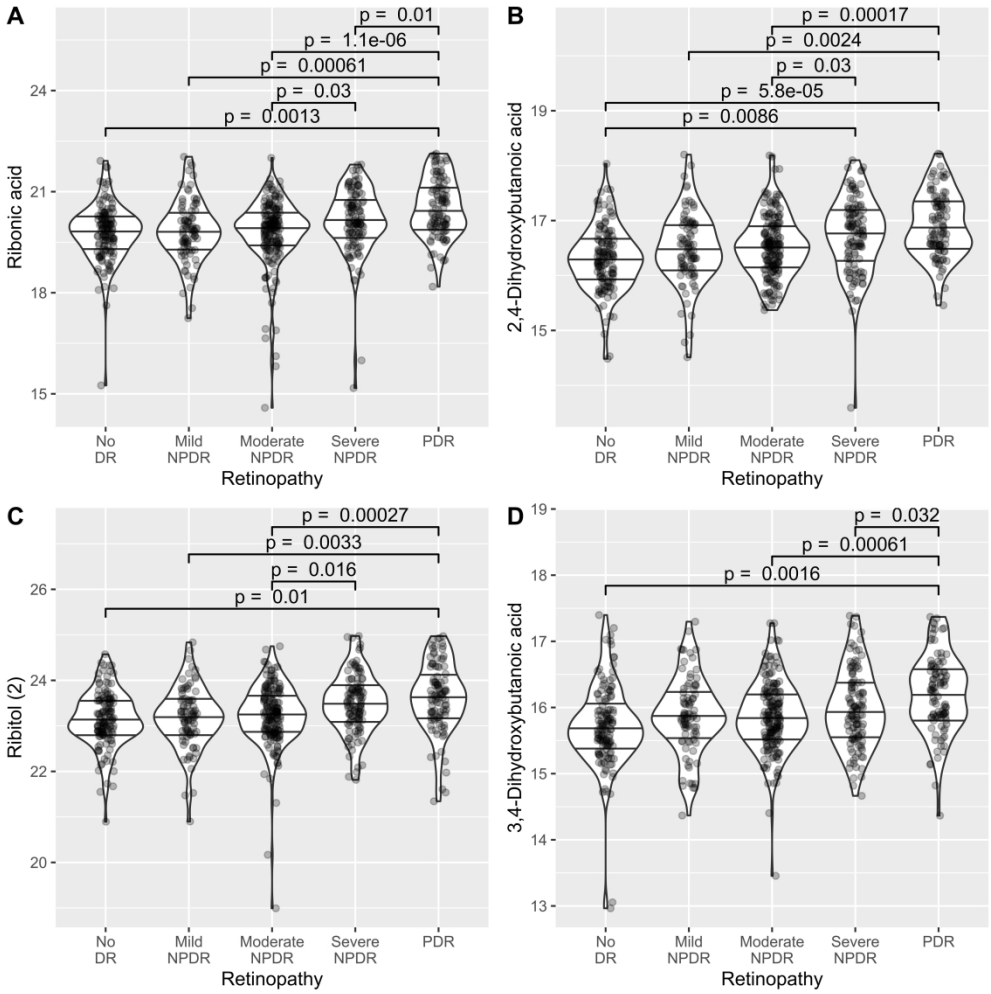

Violin plots showing metabolite levels across baseline diabetic retinopathy stages. Shown are four metabolites, where a difference between the stages was detected using ANCOVA. Observations are shown as dots and their distribution in each of the stages of retinopathy as a violin geom. Pairwise differences between the stages are indicated with p-values at the top-part of the figure. The results are from models with adjustment for age, sex, HbA1c, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. A: ribonic acid, B: 2,4-dihydroxybutyric acid, C: ribitol, and D: 3,4-dihydroxybutyric acid, HbA1c: glycated hemoglobin, DR: Diabetic retinopathy, NPDR: Non-proliferative DR, PDR: Proliferative DR

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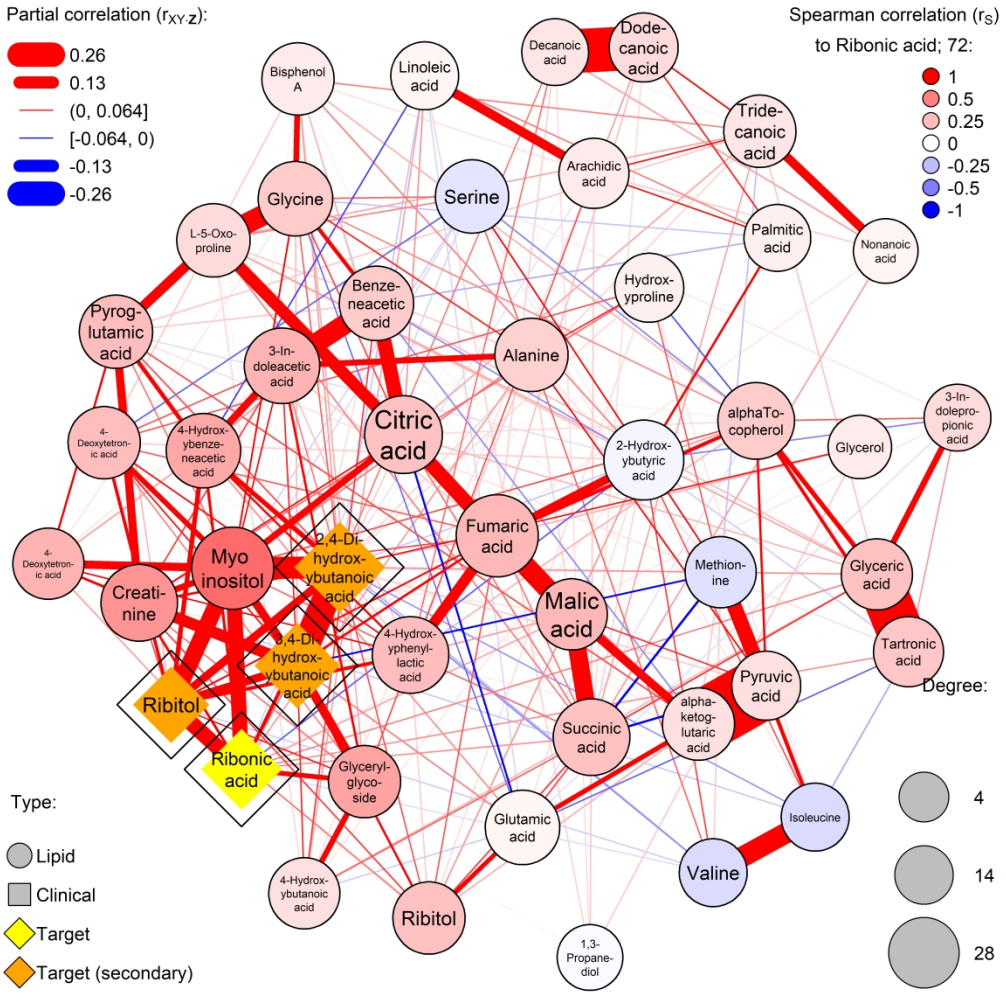

Figure 2:
Partial correlation network showing associations in the measured metabolome. Metabolites with cross-sectional association to retinopathy (ribitol, ribonic acid, 2,4-dihydroxybutyric acid and 3,4-dihydroxybutyric acid) are indicated as diamonds-shaped nodes. The node color of the other metabolites (circular nodes) indicates the Spearman correlation to ribonic acid. Partial correlations (i.e., independent associations) are indicated by lines between the metabolites, where thickness and color of the line, respectively, indicate the strength and the sign of the association (red: positive, blue: inverse). Additionally, size of the node indicates the degree of the node (i.e., the number of associations with the metabolite).

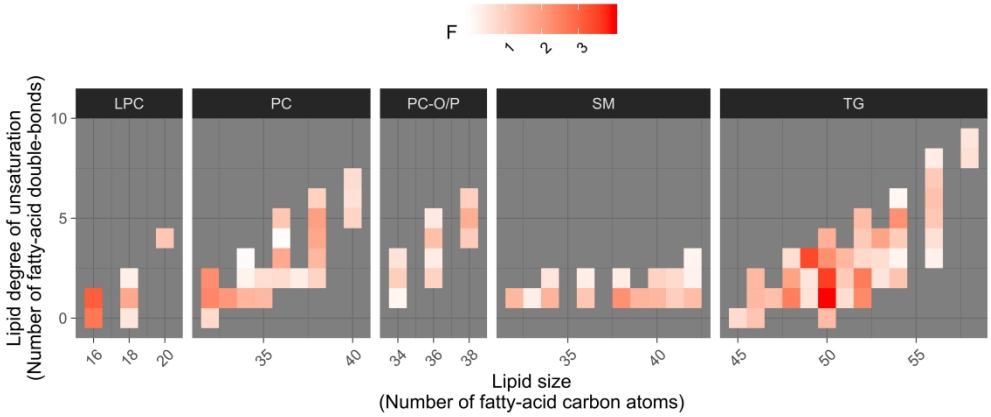

Figure 3: Heatmaps of the ANCOVA model F-statistics across the entire lipidomic panel in relation to diabetic retinopathy stage. The results are from models with adjustment for age, sex, HbA1c, systolic blood pressure, smoking, body mass index, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. Lipid species are grouped according to the lipid classes in each panel. Each cell represents one lipid species. On the y-axis is number of double-bonds for the specific species (indicating level of saturation) and on the x-axis is the number of carbon atoms (indicating total fatty acid chain length).

203x88mm (600 x 600 DPI)

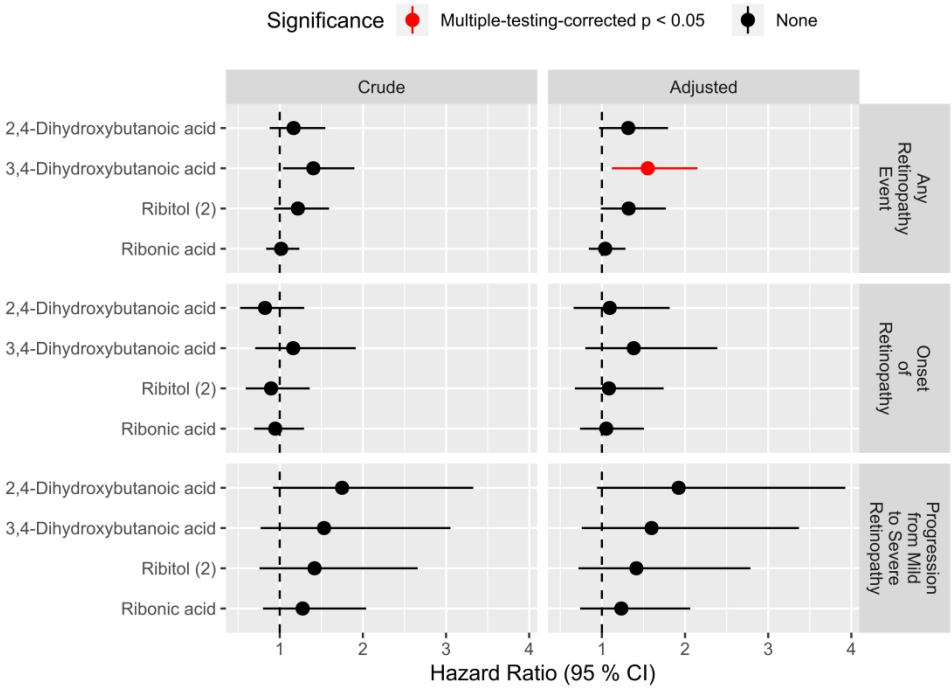

Figure 4: Forest plot of multiple-testing-corrected hazard ratios of the selected metabolites for three retinopathy events: any retinopathy, onset of retinopathy, and progression from mild to severe retinopathy (top, middle and bottom panels, respectively). Adjusted model (right) is adjusted for age, sex, HbA1c, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol and prescribed antihypertensive medication. Crude model (left) is without these adjustments.

177x127mm (600 x 600 DPI)

Supplementary Table 1: Metabolites

List of analysed metabolites.

Name
1-Dodecanol
1-Monopalmitin
1,3-Propanediol
11-Eicosenoic acid
2-hydroxy Isovaleric acid
2-Hydroxybutyric acid
2-Palmitoylglycerol
2,4-Dihydroxybutanoic acid
3-Hydroxybutyric acid
3-Indoleacetic acid
3-Indolepropionic acid
3,4-Dihydroxybutanoic acid
4-Deoxytetronic acid (1)
4-Deoxytetronic acid (2)
4-Hydroxybenzeneacetic acid
4-Hydroxybutanoic acid
4-Hydroxyphenyllactic acid
Alanine
alpha-ketoglutaric acid
alpha-Tocopherol
Aminomalonic acid
Arabinopyranose
Arachidic acid
Arachidonic acid
Benzeneacetic acid
Bisphenol A
Campesterol
Cholesterol
Citric acid
Creatinine

Decanoic acid
Docosahexaenoic acid
Dodecanoic acid
Eicosapentaenoic acid
Ethanolamine
Fumaric acid
Glutamic acid
Glyceric acid
Glycerol (1)
Glycerol (2)
Glyceryl-glycoside
Glycine
Heptadecanoic acid (1)
Heptadecanoic acid (2)
Hydroxylamine
Hydroxyproline
Isoleucine
L-5-Oxoproline
Lactic acid
Leucine
Linoleic acid
Malic acid
Methionine
Myo inositol
Myristoleic acid
Nonadecanoic acid
Nonanoic acid
Octanoic acid
Oleic acid
Palmitic acid
Phenylalanine
Proline
Pyroglutamic acid
Pyruvic acid
Ribitol (1)

Ribitol (2)
Ribonic acid
Serine
Stearic acid
Succinic acid
Tartronic acid
Threonine
Tridecanoic acid
Tyrosine
Valine

Supplementary Table 2: Lipids

List of analysed lipids.

Name
LPC(16:0)
LPC(16:1)
LPC(18:0)
LPC(18:1)
LPC(18:2)
LPC(20:4)
PC(16:0e/18:1(9Z))
PC(32:0)
PC(32:1)
PC(32:2)
PC(33:1)
PC(34:1)
PC(34:2)
PC(34:3)
PC(35:1)
PC(35:2)
PC(36:2)
PC(36:3)
PC(36:4)
PC(36:5)

PC(37:2)
PC(38:2)
PC(38:3)
PC(38:4)
PC(38:5)
PC(38:6)
PC(40:5)
PC(40:6)
PC(40:7)
PC(O-34:2)
PC(O-34:3)
PC(O-36:2)
PC(O-36:3)
PC(O-36:4)
PC(O-36:5)
PC(O-38:4)
PC(O-38:5)
PC(O-38:6)
SM(42:2)
SM(d16:1/18:1) or SM(d18:2/16:0)
SM(d18:1/24:0)
SM(d18:1/24:0) or SM(d18:0/24:1)
SM(d18:2/24:1)
SM(d32:1)
SM(d33:1)
SM(d34:1)
SM(d36:1)
SM(d36:2)
SM(d38:1)
SM(d38:2)
SM(d39:1)
SM(d40:1)
SM(d40:2)
SM(d41:1)
SM(d41:2)

TG(14:0/16:0/18:1)
TG(14:0/18:1/18:1)
TG(14:0/18:2/18:2)
TG(16:0/18:0/18:1)
TG(16:0/18:2/18:2)
TG(16:0/18:2/18:3)
TG(16:0/18:2/22:6)
TG(16:0/22:5/18:1) or TG(20:4/18:1/18:1)
TG(18:0/18:1/20:4)
TG(18:1/12:0/18:1) or TG(18:2/16:0/14:0)
TG(18:1/18:1/16:0)
TG(18:1/18:1/18:1)
TG(18:1/18:1/22:6)
TG(18:1/18:2/18:2)
TG(18:2/18:1/16:0)
TG(18:2/18:1/18:1)
TG(18:2/18:2/18:2) or TG(18:3/18:2/18:1)
TG(18:2/22:5/16:0)
TG(45:0)
TG(46:0)
TG(46:1)
TG(46:2)
TG(47:1)
TG(48:3)
TG(49:1)
TG(49:2)
TG(49:3)
TG(50:0)
TG(50:1)
TG(50:2)
TG(50:3)
TG(51:1)
TG(51:2)
TG(51:3)
TG(52:2)

TG(52:3)
TG(52:4)
TG(52:5)
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TG(53:3)
TG(53:4)
TG(54:2)
TG(54:3)
TG(54:4)
TG(54:5)
TG(54:6)
TG(56:3)
TG(56:4)
TG(56:5)
TG(56:6)
TG(56:7)
TG(58:9)